REMARKS

Claims 2, 4-11, and 53-55 are pending and under consideration. Applicants respectfully request reconsideration of the pending claims in view of the comments herein.

I. Rejection Under 35 U.S.C. § 101

Claims 2, 4 to 11 and 53 to 55 stand rejected under 35 U.S.C. § 101, for reasons of record in the prior Office Action. The Office Action maintains that there is no activity known to be associated with the encoded protein. Regarding the Applicants arguments, the Office Action asserts that that the fact that the polynucleotide is homologous to TGF-beta 4 does not endow it with a utility. The Office Action asserts that since the specification does not disclose a function for TGF-beta 4, it does not imply any activity for GDF-16. Furthermore, the Office Action asserts that sequence similarity may provide guidance as to function, but does not indicate that genes are similarly expressed.

To be useful per 35 U.S.C. § 101 an invention must be capable of some beneficial use in society. Chisum on Patents 4.02; Phillips Petroleum Co. v. U.S. Steel Corp., 673 F. Supp. 1278 (D. Del. 1987), aff'd,. 865 F.2d 1247 (Fed. Cir. 1989). A small degree of utility is sufficient to meet the utility requirement. E.I. du Pont De Nemours and Co. v. Berkley and Co., 620 F.2d 1247, 1260 n.17, 205 USPQ 1, 10 n.17 (8th Cir. 1980). To meet the utility requirement, an invention must disclose a utility that is specific, substantial, and credible. MPEP § 2107.2; See also, "Revised Interim Utility Guideline Training Materials" available at http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf. A utility is "specific" if it applies to the claimed subject matter but not to the general class of the invention. Id. A utility is "substantial" if it defines a real world use. Id. A utility is "credible" if an asserted utility is believable by a person of ordinary skill in the art based on the totality of evidence and reasoning provided. Even if a utility is not explicitly asserted, the utility requirement is met if an invention has a well-established utility (i.e., a person of ordinary skill in the art will immediately appreciate

why the invention is useful based on the characteristics of the invention) and the utility is specific, substantial, and credible.

As of the filing date of the present invention, GDF-16 had a well-established utility as a diagnostic tool for ebaf (TGF-beta 4) in detecting a cell proliferative disorder by detecting altered expression levels of ebaf (TGF-beta 4) in a variety of tumors. Kothapalli et al. disclose the sequence of ebaf, a TGF-beta family member whose expression is associated with endometrial bleeding. (*J. Clin. Invest.* 99, 2342-2350 (1997) (Exhibit A)). Tabibzadeh et al. disclose that ebaf is specifically expressed not only during endometrial bleeding, but also in certain tumors such as adenocarcinomas of the colon, ovary, and testis (*Frontiers in Bioscience* 2, a18-25, July 15, 1997, page 15, first full paragraph) (Exhibit B). Therefore, Tabibzadeh et al. conclude that ebaf is a useful tumor marker. (Id. at page 16, first paragraph).

The present invention discloses a polynucleotide and polypeptide sequence, GDF-16, that is structurally highly related to ebaf. In fact, the Examiner noted in Paper No. 17, that the ebaf polynucleotide sequence, as disclosed in U.S. Pat. No. 5,916,751 (the '751 patent), contains a region of 303 nucleotides that is 92% homologous to SEQ ID NO: 1 of the subject application. Accordingly, the GDF-16 polynucleotide of the present invention is useful for detecting tumors in which ebaf is specifically expressed, including adenocarcinomas of the colon, ovary, and testis. Accordingly, Applicants respectfully assert that the GDF-16 polynucleotide recited in the pending claims has a well-established utility that would have been recognized by a skilled artisan as of the filing date of the present invention.

The fact that the present invention had a well-established utility is further supported by the "Revised Interim Utility Guideline Training Materials" (referred to herein as "The Utility Guidelines) available at http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf. Example 12 of The Utility Guidelines indicates in the Caveat section, that a monoclonal antibody has a well-established utility if a receptor bound by the monoclonal antibody is present on the cell membranes of melanoma cells but not on the cell membranes of normal skin cells. This is analogous to the present situation in which the pending claims are directed at polynucleotides

that bind a polynucleotide encoding a protein, ebaf, that is specifically expressed in certain tumors. Furthermore, Example 5 of The Utility Guidelines concludes that a claimed invention directed at a partially characterized protein may have a well-established utility, where it is determined that increased levels of protein X are indicative of a disease such as heart disease. This is similar to the present situation in which increased levels of binding of the GDF-16 polynucleotide to ebaf mRNA are indicative of the presence of a tumor.

The pending claimed invention not only has a well-established utility, the specification explicitly asserts a number of related utilities for GDF-16. For example, the present specification indicates that because GDF-16 is a TGF-beta family member, it is likely that it is associated with cell growth and differentiation activity and a marker for a cell proliferative disorder (see page 3, lines 2-4; page 4, lines 1-8). Furthermore, the specification associates the GDF-16 polynucleotide with diagnosing and treating a cell proliferative disorder (see, page 19, lines 3-10), for example, a malignant cell proliferative disorder (see page 15, lines 17-24). More specifically, the specification discloses that a GDF-16 polynucleotide can be utilized in detecting and diagnosing a cell proliferative disorder by detecting an altered expression level as compared with that of a normal cell (page 19, lines 3-10). Furthermore, the specification discloses that a GDF-16 polynucleotide can be used to detect a close family member of GDF-16 (page 7, lines 18-23). Therefore, the specification asserts a number of specific and substantial utilities for GDF-16 polynucleotides related to the well-established utility discussed above.

The credibility of the asserted utilities for GDF-16 is supported by the published literature. First there is a plethora of literature available that indicate that TGF-beta family members have the utilities expressly asserted in the application. Furthermore, based on the Kothapalli et al. and Tabibzadeh et al. references cited above, and the '751 patent, one skilled in the art viewing the subject application, reasonably would conclude that a polynucleotide of the present invention encoding GDF-16 can be useful in the detection and early diagnosis of a malignant cell proliferative disorder, by using the GDF-16 polynucleotide as a probe for

expression of ebaf (TGFβ-4), as discussed above. Therefore, the art establishes the credibility of a substantial and specific utility that is asserted in the specification.

In summary, it is respectfully submitted that GDF-16 had a well-established utility as of the filing date of the present invention. Furthermore, the specification asserts specific, substantial, and credible utilities for a GDF-16 polynucleotide that are substantiated by published literature. Accordingly, it is respectfully requested that the rejection of claims 2, 4 to 11 and 53 to 55 under 35 U.S.C. § 101 as allegedly lacking utility be removed.

II. Rejection Under 35 U.S.C. § 112

Claims 2, 4 to 11, and 53 to 55 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. Applicants respectfully traverse the rejection. The Office Action maintains the rejection of the previous Office Action that the rejected claims are not enabled because there is allegedly no utility associated with the claimed polynucleotides.

As indicated above, Applicants respectfully assert that a GDF-16 polynucleotide had a well-established utility as of the filing date of the present application, of being useful in detecting the structurally highly related tumor marker, ebaf. Furthermore, as indicated above, the specification asserts a number of utilities that are specific, substantial, and credible, including that a GDF-16 polynucleotide can be utilized in detecting and diagnosing a cell proliferative disorder by detecting an altered expression level of a highly related protein, as compared with that of a normal cell. Therefore, the present specification teaches a skilled artisan how to use the invention. Accordingly, it is respectfully requested that the rejection of claims 2, 4 to 11, and 53 to 55, under 35 U.S.C. § 112, first paragraph, be removed.

In view of the amendments and the above remarks, it is submitted that the claims are in condition for allowance, and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicants' representative if there are any questions relating to this application.

Please charge any additional fees, or make any credits, to Deposit Account No. 50-1355.

Respectfully submitted,

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Detection of *ebaf*, a Novel Human Gene of the Transf rming Growth Factor β Superfamily

Association of Gene Expression with Endometrial Bleeding

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Abstract

Human endometrium is unique since it is the only tissue in the body that bleeds at regular intervals. In addition, abnormal endometrial bleeding is one of the most common manifestations of gynecological diseases, and is a prime indication for hysterectomy. Here, we report on a novel human gene, endometrial bleeding associated factor (*ebaf*), whose strong expression in endometrium was associated with abnormal endometrial bleeding. In normal human endometrium, this gene was transiently expressed before and during menstrual bleeding. In situ hybridization showed that the mRNA of *ebaf* was expressed in the stroma without any singificant mRNA expression in the endometrial glands or endothelial cells. The predicted protein sequence of *ebaf* showed homology with and structural features of the members of TGF-β superfamily. Fluorescence in situ hybridization showed that the *ebaf* gene is located on human chromosome 1 at band q42.1. Thus, *ebaf* is a novel member of the TGF-β superfamily and an endometrial tissue factor whose expression is associated with normal menstrual and abnormal endometrial bleeding. (*J. Clin. Invest.* 1997. 99:2342–2350.) Key words: human • endometrium • endometrial bleeding • ebaf • TGF-β

Introduction

Normal menstruation, a unique process of endometrial tissue shedding and bleeding, is a complex phenomenon that occurs during the reproductive years in women, some higher primates, and two nonprimate species; the elephant shrew (*Elephantus myuras jamesoni*) and the bat (*Glossophaga sorcinina*) (1). This process is associated with breakdown and loss of upper layers of endometrium (functionalis) consisting of glands and their surrounding stroma after a normal ovarian cycle. The role of steroid hormones as the primary and systemic force that drives the endometrium through the exquisitely orchestrated phases of the menstrual cycle is well recognized (2, for recent reviews see references 3–5). The elegant studies of Markee demonstrated that withdrawal from steroid hormones is responsible for the menstrual shedding of endometrium and bleeding (2). Any aberration in the priming of endometrium by estrogen followed by progesterone leads to premature and sometimes excessive bleeding during the menstrual cycle or during menstruation. This abnormal endometrial bleeding is one of the most common disorders in women, and is one of the primary causes for hysterectomy (6). During the reproductive years, nearly 20% of women exhibit menorrhagia (excessive blood loss during menstruation) (7, 8), and almost every woman during her lifetime experiences episodes of abnormal endometrial bleeding (6). The primary conditions that are associated with deranged priming of endometrium by the steroid hormones include aberrant follicular maturation, ovulation or development of corpus luteum (6, 9, 10), and administration of steroid hormones, including progestagens (9, 11–13). The underlying basis for dysfunctional uterine bleeding, a common gynecological disorder that affects women during the reproductive years, is also due to an inappropriate exposure of endometrium to steroid hormones. In most cases, this condition is almost always associated with anovulatory cycles, and occurs from a proliferative endometrium (6, 10). More than 75% of abnormal endometrial bleeding in adolescents is dysfunctional uterine bleeding due to immaturity of the hypothalamic-pituitary-ovarian axis. As a result of lack of lutenizing hormone surge and anovulation, the endometrium is continuously exposed to estrogen, and the progesterone-dominated secretory phase does not develop (10). The dysfunctional uterine bleeding in perimenopausal women has a similar basis. The aging ovary does not produce an adequate amount of estrogen to induce the midcycle surge of lutenizing hormone. In these adolescents and perimenopausal women, the endometrium is continuously exposed to estrogen, and bleeding is thought to occur as a result of this unopposed estrogen exposure (9). On the other hand, dysfunctional uterine bleeding may occur during the secretory phase, due to, for example, an inadequate luteal phase (9). Bleeding which occurs as spotting or breakthrough-bleeding, or occasionally as heavy bleeding, may happen while the steroid hormones are being administered as a therapeutic measure or for contraception (9, 11–13). Despite the knowledge that steroid hormones are responsible for normal and abnormal endometrial bleeding, the identity of the local and specific endometrial factors which are implicated remain largely unrecognized. Endometrium is a unique tissue,

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since other tissues, such as breast, despite the expression of steroid hormone receptors and response to these hormones, do not bleed at the time when the serum levels of steroid hormones have fallen and endometrium is menstruating. This fact suggests that there should be tissue-specific factors that make endometrium susceptible to bleeding. Here, we report on a novel human gene of the TGF-β superfamily whose expression in endometrium is confined to both the late secretory phase and during endometrial bleeding.

Methods

Materials. The differential display kit (RNA image kit) was obtained from GenHunter Corporation (Brookline, MA). pBluescript® SK⁻ and human placental cDNA library were purchased from Stratagene Inc. (La Jolla, CA). A 1.1-kb cDNA fragment of GAPDH (glyceraldehyde 3-phosphate dehydrogenase)[1] was obtained from Clontech (Palo Alto, CA). Reagent kit for sequencing with sequenase and Hybond nylon membrane were from Amersham Corp. (Arlington Heights, IL). Deoxycytidine 5′ triphosphate dCTP α-^{32}P (3,000 Ci/mmol) was from Dupont-NEN (Boston, MA). Prime-a-Gene labeling kit was from Promega Corp. (Madison, WI). RNA STAT-60™ was from Tell-Test, Inc. (Friendswood, TX). Silane-coated, RNAse-free slides coated (Silane-Prep™) for in situ hybridization and the Kodak-OMAT films were obtained from Sigma Chemical Company (St. Louis, MO). Nick columns were obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Digoxygenin labeling kit (SP6/T7) and DIG nucleic acid detection kit were from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were from either Sigma Chemical Company or Fisher Scientific Co. (Pittsburgh, PA).

Processing of endometria. Endometrial tissues were obtained as biopsy or curettings from hysterectomy specimens of normal fertile women who underwent these procedures for nonendometrial abnormalities such as ovarian or cervical lesions. In addition, endometrial samples were obtained from a group of patients with endometrial bleeding. The age, menstrual cycle history, date of endometrium, type of bleeding, and other associated clinical findings in these patients are presented in Table I. The endometria of these patients, consistent with anovulatory and ovulatory cycles exhibited a proliferative and secretory pattern, respectively. In patients whose endometrium had a proliferative pattern, bleeding was the major reason for hysterectomy (see Table I). Hysterectomy specimens and each endometrial biopsy sample were rapidly processed. The date of endometrium was determined based on the morphologic evaluation of hematoxylin and eosin-stained endometrial sections using established criteria of Noyes and Hertig (14). Each endometrial sample was aliquoted as required. Most tissues, however, were used as follows: ~ 10% of each sample was processed for paraffin sectioning and morphologic examination, ~ 70% was flash frozen in a dry ice/ethanol bath for isolation of RNA; and the remaining 20% was frozen in OCT mounting medium (Tissue-Tek II; Miles Laboratories, Inc., Naperville, IL) for cryostat sectioning and in situ hybridization studies.

Differential display. Differential display was performed as described (15, 16). Briefly, cDNA synthesis was carried out using an anchored oligo-(dT). The quality of cDNA was verified by PCR using gene-specific primers to actin which would produce a specific product of defined length detectable by agarose gel electrophoresis. The templates for differential display consisted of cDNAs prepared from total RNAs isolated from endometria from various phases of the menstrual cycle. These included two cases of proliferative endometria, three cases of postovulatory day 5 endometria, one case of postovulatory day 12 endometrium, one case of postovulatory day 14 endometrium, and one case of menstrual endometrium. The cDNAs were amplified with several primer sets. The amplified products from different cDNAs were size-fractionated on a denaturing polyacrylamide gel. Several amplified products were differentially expressed throughout the menstrual cycle. The primer set consisting of an anchored oligo-(dT) (AAG CTT TTT TTT TTT C) and an arbitrary primer (AAG CTT GAT TGC C) led to the identification of two differentially expressed PCR products. One of these products, present in the late secretory menstrual endometria, was removed from the sequencing gel from the lane that contained the amplified products from postovulatory day 12. This band was reamplified, and was run on a sequencing gel along with the amplified product of the postovulatory day 12 sample. The mobility of the reamplified band was the same as that seen in the amplified product from the postovulatory day 12 sample. The cDNA of the reamplified product was cloned using a T-tailed vector (pBluescript II KS⁻) (17, 18). To assure that the cloned product is derived from the same gene, seven independent clones were sequenced (19), and were found to contain an identical 300-bp sequence.

Isolation of RNA and Northern blotting. The RNA was extracted by using acid guanidinium thiocyanate-phenol-choloroform extraction method as described (20). Briefly, the tissues were homogenized in RNA STAT-60™. Each 50–100 mg of tissue was homogenized in 1 ml of RNA STAT-60™ in a glass or Teflon Dounce homogenizer. Each homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added for each milliliter of RNA STAT-60™ used. Each sample was covered and shaken vigorously for 15 s, and allowed to stand at room temperature for 2–3 min. After centrifugation at 12,000 *g* for 15 min at 4°C, each homogenate was separated into a lower phenol/chloroform phase and an upper aqueous phase. RNA in the upper aqueous phase was transferred to fresh tubes and mixed with isopropanol to precipitate the total RNA. After centrifugation and drying, the precipitated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by vigorous pipetting and by a gentle heating at 55–60°C. The amount of RNA in each sample was determined spectrophotometrically, and its quality was evaluated by the integrity of ribosomal RNA by electrophoresis of 20 μg of total RNA in 1% formaldeyde-agarose gel in the presence of ethidium bromide. Northern blotting was done as described (21). Briefly, 20 μg of total RNA of each sample was denatured at 65°C in a RNA loading buffer, electrophoresed in 1% agarose containing 2.2 M formaldehyde gel, and blotted onto a Hybond nylon membrane using a positive pressure transfer apparatus (Posiblot; Stratagene Inc.). The RNA was fixed to the membrane by UV crosslinking. Using the Prime-a-Gene kit, cDNA was labeled with [^{32}P] to a high specific activity, and purified by Nick columns. Membranes were prehybridized in 50% formamide, 10× Denhardt's solution, 4% saline sodium citrate (SSC), 0.05 M sodium pyrophosphate, and 0.1 mg/ml of denatured herring sperm DNA at 42°C for 2–4 h and hybridized for 16 h at 42°C with 10^6 cpm/ml of heat-denatured probe in the same buffer containing 10% dextran sulphate. Then, membranes were sequentially washed three times in 4× SSC, one time in 0.5× SSC, and then one time in 0.1× SSC. All washes contained 0.1% sodium dodecyl sulphate (SDS), and were done at 65°C for 20 min each. The membranes were subjected to autoradiography at −70°C with intensifying screens. The same blot was stripped and reprobed for GAPDH. To reprobe a blot, the probe was stripped from the membrane in 75% formamide, 0.1× saline sodium phosphate ETDA (SSPE), and 0.2% SDS at 50°C for 1 h. The relative abundance of mRNA in each band in the autoradiograms was quantitated by laser scanning densitometry. The relative optical densities of the GAPDH bands were used to normalize the relative optical densities of the bands from the *ebaf* mRNA.

In situ hybridization. Digoxigenin-labeled sense and antisense RNAs of *ebaf* were synthesized by in vitro transcription of the full-length cDNA cloned into pBluescript® SK⁻ using digoxygenin dUTP. After alkaline hydrolysis, the probes were subjected to agarose gel electrophoresis to determine the size of the digested RNA fragments.

1. *Abbreviation used in this paper:* GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

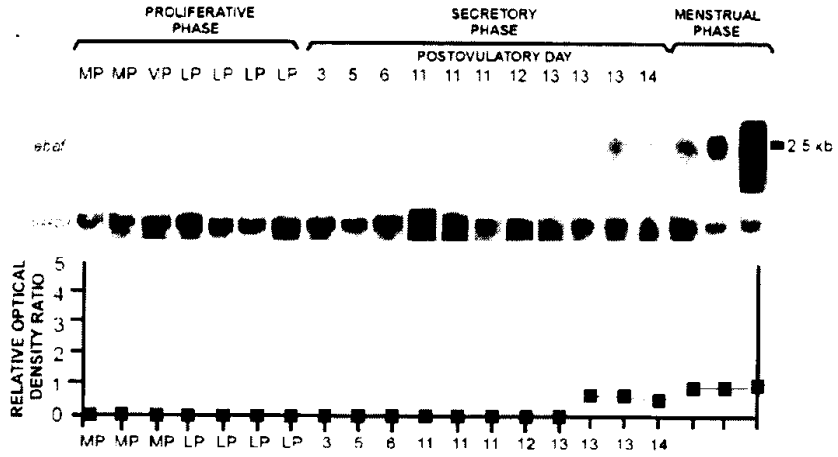

Figure 1. Northern blot analysis of *ebaf* in human endometrium throughout the menstrual cycle. (*Top*) 20 μg total RNA from each endometrium was subjected to Northern blot analysis using a 300 bp at the 3′ end of the *ebaf* cDNA, or the entire cDNA as the probe. As shown, a band of mRNA in the size of 2.5 kilobase (kb) was detected only in the late secretory and menstrual endometria. The *ebaf* mRNA was not detectable in endometrium during the proliferative phase, or in early (postovulatory days 1–5) or midsecretory (postovulatory days 6–8) phases of the menstrual cycle. The integrity of RNA and equal loading was verified by staining the 18s and 28s ribosomal RNAs (not shown) and hybridization of the blots with a probe to GAPDH. *MP*, mid proliferative; *LP*, late proliferative. (*Bottom*) The relative optical densities of the *ebaf* and GAPDH mRNA bands were determined by laser scanning densitometry. The bottom panel shows the relative optical density ratios of the *ebaf*/GAPDH bands.

Dot blotting was performed on the RNA fragments to assure that they were labeled. In situ hybridization was performed as previously described (22, 23). Briefly, frozen sections of endometria were mounted on silane-coated RNAse-free slides, and fixed in 4% formalin in PBS for 15 min at 4°C. The tissue sections were rinsed in 2× SSC and then treated with proteinase K (1 μg/ml in 0.1 M Tris, 50 mM EDTA, 20 min, 37°C) and acetylated for 10 min in 0.1 M triethanolamine (pH 8.0), 0.9% sodium chloride, and 0.25% acetic anhydride. The slides were dipped once in 2× SSC, and then were dehydrated in ascending series of ethyl alcohol, and air-dried. The slides were prehybridized for 1 h at 37°C in 50% formamide, 1× Denhardt's solution, and 500 μg/ml tRNA, 0.3 M sodium chloride, 10 mM Tris, 1 mM EDTA (pH 8), and 10% dextran sulfate. Then, sections were incubated at 55°C overnight in the same solution containing the appropriate concentration of the probe. The amounts of labeled probes needed were empirically determined first by a series of in situ hybridization experiments using various dilutions of the probes. Sense probe was used as the control. After hybridization, slides were washed three times for 10 min each at room temperature in 2× SSC, and the excess SSC was removed. The sections were then incubated with RNAse A (20 μg/ml) in 500 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 8) at 37°C for 30 min to remove the nonhybridized RNA. The sections were washed three times at room temperature, for 15 min each, in 2× SSC, 1× SSC, and 0.5× SSC and a final wash in 0.1× SSC at 55°C for 45 min. Slides were washed in 100 mM Tris (pH 8), 150 mM sodium chloride for 10 min. Then, the sections were blocked in 5% normal horse serum in the same buffer for 20 min at 37°C. Slides were incubated with alkaline phosphatase–labeled, anti-digoxygenin antibody for 1 h at 37°C, washed, and developed in a mixture of Nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Metaphase cell preparation and fluorescence in situ hybridization (FISH). Metaphase spreads were obtained from PHA-stimulated lymphocytes of normal human peripheral blood. G-banding was performed on air-dried slides after they were aged for 1 wk. The banded metaphase chromosomes were examined and photographed with a MAX-BX 40 Olympus microscope using a UWplan FL × 100 objective (dry lens). The coordinates of each metaphase were recorded. The slides were then destained with two changes of 3:1 methanol/acetic acid for 5 min each, and air-dried. The full length *ebaf* cDNA was used as the probe in the FISH.

Results and Discussion

Identification of ebaf by differential display. We were interested in testing the hypothesis of whether gene(s) exist whose expression in endometrium is confined to the premenstrual/menstrual period. We reasoned that the genes that are involved in the process of menstrual bleeding potentially will be expressed immediately before and during menstruation. To test the hypothesis, we used differential display and looked for gene(s) whose expression in endometrium was confined to the late secretory/menstrual phase. We identified a major band in the differential display gel whose expression, consistent with our hypothesis, was found in postovulatory days 12 and 14 and the menstrual endometria. A GenBank nucleotide BLAST search revealed the sequence of this band to be 100% identical to the expressed sequence tag (EST) cDNA clone 137335 3′ that has been derived from human placenta. To obtain the full-length coding sequence of this gene, we screened a human placental

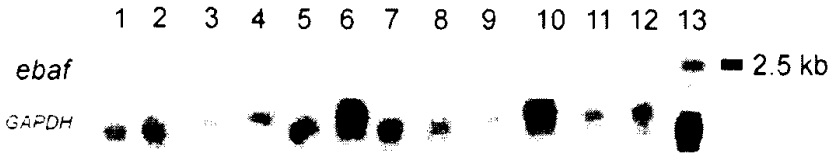

Figure 2. Northern blot analysis of *ebaf* mRNA expression in normal tissues. (*Top*) 20 μg total RNAs from normal tissues was subjected to Northern blot analysis using the full-length *ebaf* cDNA as the probe. The integrity of RNA was verified by staining the 18S and 28S ribosomal RNA (not shown) and hybridization of the blots with a cDNA probe to GAPDH. The tissues in lanes *1–12* were, respectively, as follows: lung, kidney, ovary, liver, colon, rectum, spleen, lymph node, pancreas, testis, stomach, and stomach mucosa. Lane *13* is a positive control RNA sample from endometrium of a patient with endometrial bleeding showing the *ebaf* mRNA band (see Fig. 4). Whereas GAPDH mRNA was detected in these tissues, the *ebaf* mRNA was not detectable in tissues other than endometrium.

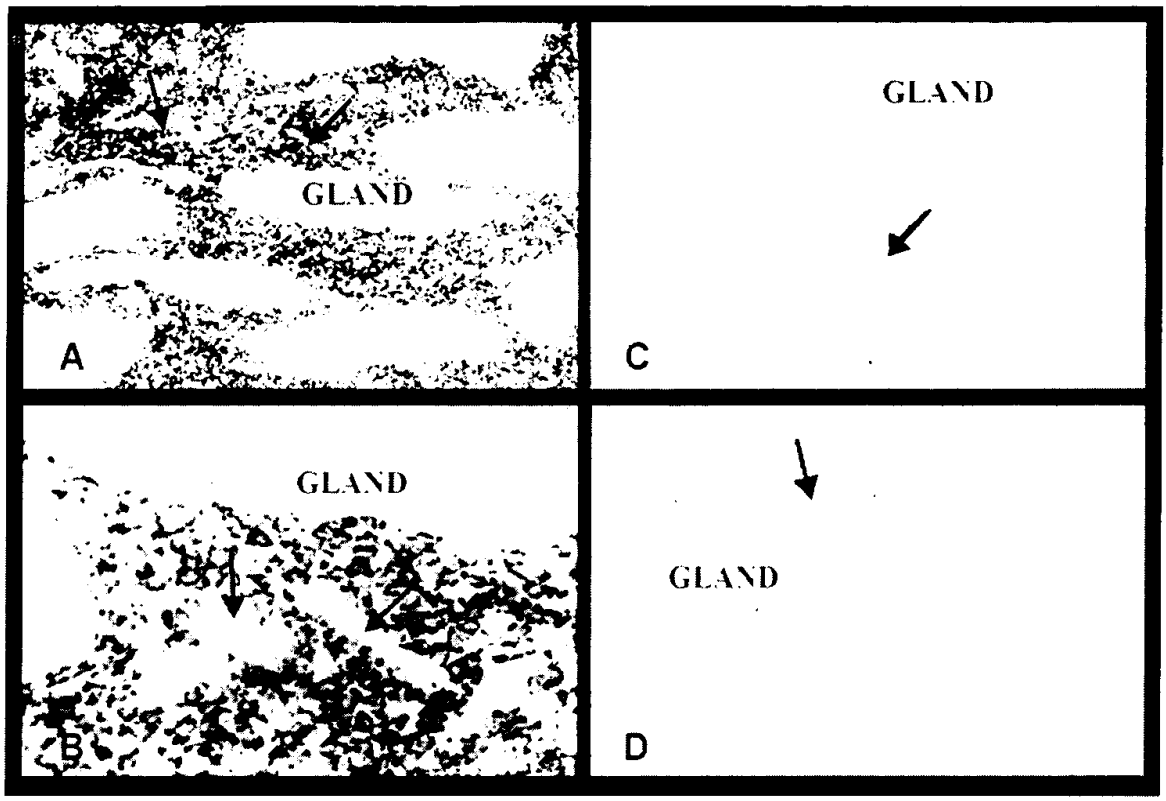

Figure 3. In situ hybridization of *ebaf* mRNA in normal human endometrium. Sections of late secretory endometria exhibited hybridization signals with digoxygenin-labeled, antisense, *ebaf* RNA probe in the stroma (*A*, ×50; *B*, > 200). On the other hand, sections of late proliferative endometria failed to show any hybridization signal with the antisense *ebaf* RNA probe (*C*, ×50; *D*, ×200). Endothelial cells did not exhibit hybridization signals with the digoxygenin-labeled, antisense *ebaf* RNA in secretory (*A* and *B*) or proliferative (*C* and *D*) endometria (*arrows*).

library with the 300-bp fragment. Three independent clones with a 2.0-kb cDNA insert were identified and sequenced in both directions, and cDNA of one of these clones (*ebaf*) was used in the following studies.

Expression of ebaf mRNA in normal human endometrium throughout the menstrual cycle. Northern blot analysis showed that in normal human endometrium, the expression of the *ebaf* mRNA was menstrual cycle phase-specific. The *ebaf* mRNA in human endometrium was transiently expressed in the late secretory/menstrual phase (Fig. 1). The size of the *ebaf* mRNA species detected in all endometria was ~ 2.5 kb (Fig. 1). The expression of *ebaf* mRNA could not be detected in endometria

Table I. Characteristics of Patients with Endometrial Bleeding

Case no.	Age	Menstrual cycle history		Date of endometrium	Characteristics of bleeding	Other clinical findings
		Regular	Irregular			
1	40		Y	P	Menometrorrhagia*	Endometriosis
2	37	Y		P	Menorrhagia* (2 yr)	
3	34	Y		P	Menorrhagia*	
4	35	Y (8–12 d)		P	Menorrhagia*	Ovarian cyst, adhesions
5	39		Y	P	Menometrorrhagia*	Adenomyosis
6	28		Y (8–9 d)	P	Menometrorrhagia*	CINIII
7	45		Y	P	Severe menometrorrhagia, uncontrolled*	Adenomyosis, pelvic pain
8	36	Y		MS	Menometrorrhagia	Leiomyoma
9	43	Y		ES	Menometrorrhagia	Leiomyoma
10	22	Y		MS	Menometrorrhagia	CINIII
11	44	Y		MS	Menometrorrhagia	Ovarian cyst
12	43	Y		ES	Menometrorrhagia	Leiomyoma

Y, yes; *P*, proliferative; *ES*, early secretory; *MS*, midsecretory; *LS*, late secretory. *Endometrial bleeding was the major reason for hysterectomy.

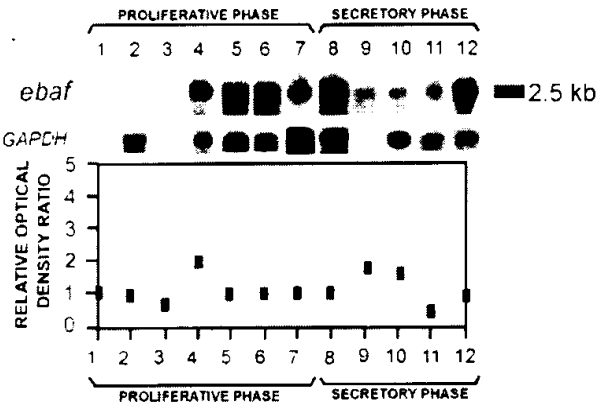

Figure 4. Northern blot analysis of *ebaf* mRNA expression in endometria of patients with endometrial bleeding. (*Top*) 20 μg total RNAs from endometria of patients with endometrial bleeding was subjected to Northern blot analysis using the full-length *ebaf* cDNA as the probe. The integrity of RNA was verified by staining the 18s and 28s ribosomal RNA (not shown) and hybridization of the blots with a cDNA probe to GAPDH. The endometria were dated to the proliferative, early, and midsecretory phases. The mRNA of *ebaf* is strongly expressed in all endometria irrespective of whether the endometrium was in the proliferative or secretory phase of the menstrual cycle. In most patients with endometrial bleeding, no organic lesions that could account for the bleeding were detected. Three patients had leiomyomata (lanes *8,9*, and *12* from left). (*Bottom*) The relative optical densities of the *ebaf* and GAPDH mRNA bands were determined by laser scanning densitometry. The bottom shows the relative optical density ratios of the *ebaf*/GAPDH bands.

in the proliferative or early and midsecretory phases of the menstrual cycle (Fig. 1). Northern blot analysis failed to reveal the presence of 2.5 kb *ebaf* mRNA in a number of normal tissues including lung, kidney, ovary, liver, colon, rectum, spleen, lymph node, pancreas, testis, and stomach (Fig. 2)

In situ hybridization confirmed the findings of Northern blot analysis. Sections of late secretory endometria exhibited hybridization signals with the antisense *ebaf* RNA probe in the stroma. Within stroma, the mRNA expression was confined to predecidualized stromal cells in the upper layers of endometrium underlying the surface epithelium (Fig. 3, *A* and *B*). In the same endometria, the stroma in the basal part of the endometrium overlying the myometrium failed to exhibit evidence of mRNA expression (data not shown). In these endometria, with exception of a few glands located near the surface epithelium, virtually no *ebaf* mRNA expression could be identified in the epithelial cells On the other hand, sections of late proliferative endometria failed to show any hybridization signal with the antisense *ebaf* RNA probe (Fig 3, *C* and *D*). Endothelial cells (*arrows*, Fig. 3, *A–D*) did not exhibit hybridization signals with the antisense *ebaf* probe. All endometria exhibited hybridization signals with a digoxygenin-labeled antisense GAPDH RNA probe, but not with the sense *ebaf* RNA probe (data not shown). This finding suggests that expression of *ebaf* mRNA occurs primarily in stromal cells that have been predecidualized. The Northern blot and in situ hybridization data suggest that the expression of *ebaf* in the human endometrial stroma may be considered as part of the premenstrual/

Table II. The Extent of Identity and Similarity Between the ebaf and Other Members of the TGF-β Superfamily

Name of gene	% Identity	% Similarity
ebaf	100	100
lefty	77	83
MIS	26	68
TGF-β_3	25	65
BMP-7	25	64
TGF-β_1	23	65
INH-β B chain	23	63
BMP-3	23	64
GDF-9	22	64
GDF-3	21	60
INH-β A chain	20	65

The deduced amino acid sequence of *ebaf* was aligned with the amino acid sequences of various members of the TGF-β superfamily using the CLUSTAL program, and the percent identities and similarities were calculated (32). Except for lefty, GDF-3, and GDF-9, all other sequences were from humans. *ebaf*, endometrial bleeding associated factor; *MIS*, mullerian inhibiting factor precursor; *TGF-β_1*, transforming growth factor β_1 precursor; *BMP-7*, bone morphogenetic protein-7 precursor; *TGF β_3*, transforming growth factor β_3 precursor; *INH-β A*, inhibin-β A chain precursor; *GDF-9*, growth differentiation factor-9; *GDF-3*, growth differentiation factor-3.

menstrual molecular repertoire which is expressed before and during menstrual shedding.

Expression of ebaf mRNA in endometria of patients with endometrial bleeding. The findings suggested that the *ebaf* expression may be closely associated with endometrial bleeding. To validate this hypothesis, endometria were obtained from a group of patients with endometrial bleeding. Dating of these endometria showed some to be in the proliferative phase, and others to be in the secretory phase (Table I). In most patients, organic lesions that could account for the endometrial bleeding could not be found. In three patients, however, the endometrial bleeding was associated with the presence of uterine leiomyoma (Table I). The mRNA of *ebaf* was strongly expressed in endometria of these women (Fig. 4). Interestingly, the expression of mRNA of *ebaf* was observed in endometrium regardless of whether the bleeding endometrium was in the proliferative, early, or midsecretory phases of the menstrual cycle when no expression of the gene was expected. These findings validate the hypothesis that endometrial bleeding is associated with the expression of *ebaf* mRNA in human endometrium.

Characterization of full-length ebaf cDNA. In view of these findings, the clones containing the longest cDNAs of *ebaf* were isolated from a placental library and sequenced (19). Within the *ebaf* cDNA, a consensus polyadenylation signal was present eight bases upstream from the poly-A tail (Fig. 5). GenBank search revealed that the deduced amino acid sequence of ebaf shows a great amount of identity and similarity with the known members of the TGF-β superfamily (Table II). The predicted protein sequence of ebaf showed a strong homology to the mouse lefty/stra3 (24, 25). The deduced amino acid sequence of ebaf protein is 77% identical and 83% similar to lefty protein (Fig. 6). A motif search revealed that the pre-

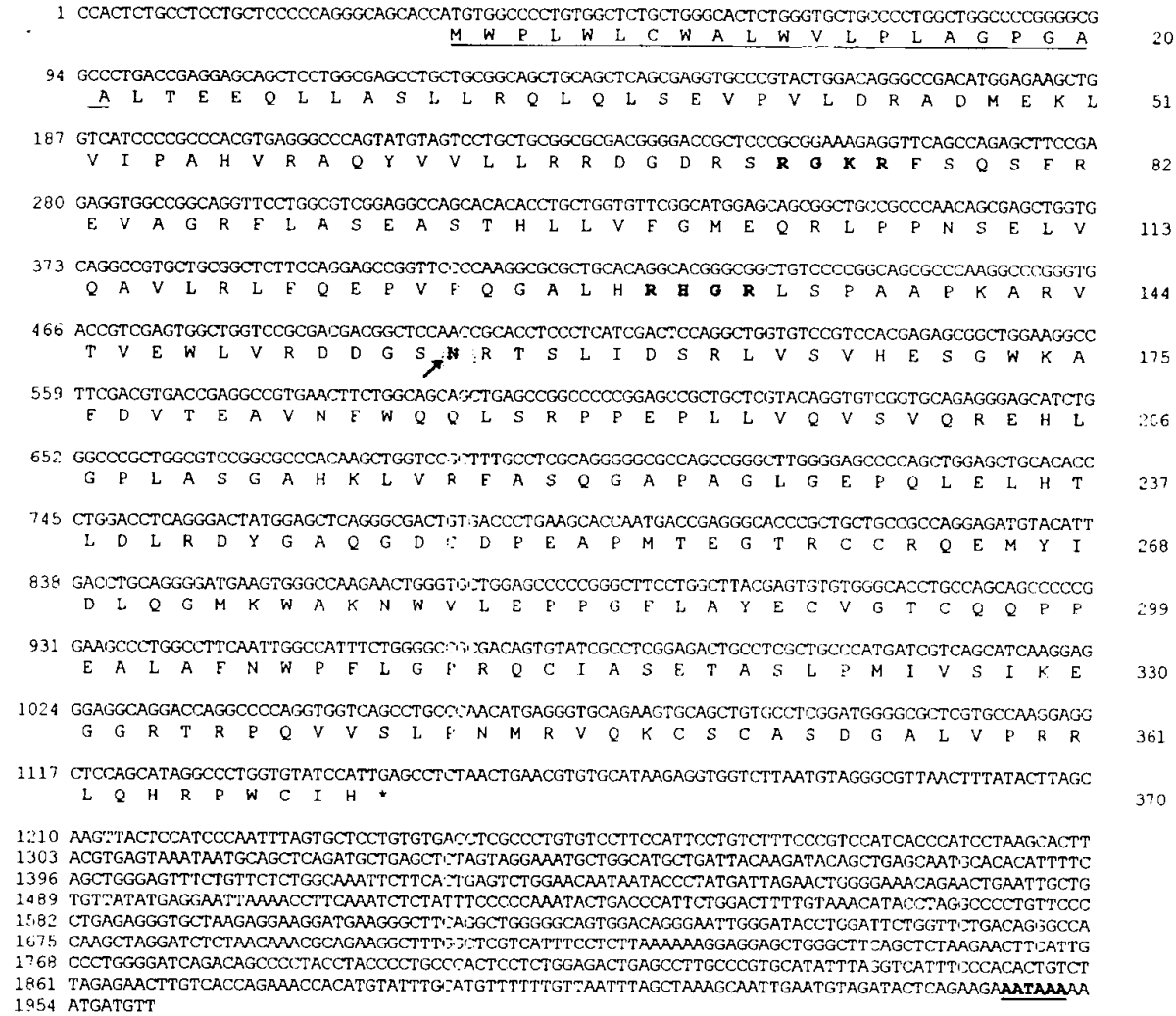

Figure 5. Nucleotide and deduced amino acid sequences of *ebaf.* The nucleotide sequence of *ebaf* contained an open reading frame with an initial methionine codon that conformed to the Kozak consensus (GCACCATG) sequence (32). The potential signal peptide is underlined, the putative tetrabasic processing sites (**RGKR** and **RHGR**) are shown in bold, and the potential glycosylation site is circled (*arrow*). A consensus polyadenylation signal (**AATAAA**) was present eight bases upstream from the poly-A tail of *ebaf* cDNA. The sequence was deposited in GenBank database under accession number; Bankit8464[illegible] JU81523.

dicted ebaf protein contains most of the cysteine residues which are conserved among the TGF-β–related proteins (26), and which are necessary for the formation of the cysteine knot structure (27, 28) (Fig. 7). The ebaf sequence contains an additional cysteine residue, 12 amino acids upstream from the first conserved cysteine residue. The only other family members known to contain an additional cysteine residue are TGF-βs, inhibins, and GDF-3 (26, 29). ebaf, similar to lefty, GDF-3/Vgr2, and GDF-9, lacks the cysteine residue that is known to form the intermolecular disulfide bond (29, 30). Therefore, ebaf appears to be an additional member of the TGF-β superfamily with an unpaired cysteine residue that may not exist as a dimer. Nevertheless, it has been suggested that, in GDF-3 and GDF-9 (which also lack such a cysteine residue) hydrophobic contacts between the two monomer subunits may promote dimer formation (26). Whereas the carboxy terminus of the TGF-β family is usually CX1CX1, ebaf has a longer COOH-terminal sequence, CX1CX19 (Fig. 5). In addition, the members of the TGF-β superfamily are synthesized as preproproteins, which are cleaved at RXXR site to release the mature form of the protein. The predicted protein of ebaf exhibits two such RXXR sites that are located at amino acid residues of 73-76 and 131-134, respectively (Fig. 5). If one of these sequences is the cleavage site, a mature protein of 294 and 236 amino acids should be produced. The deduced amino acid sequence of lefty also contained two potential cleavage sites at amino acid residues of 74-77 and 132-135 with mature proteins of 291 and 233 amino acids (24). In the case of lefty, the expression of the protein in 293T cells led to formation of a nonsecretory, 42-kD protein which is the size of the preproprotein (24). Expression in BALB/3T3 cells, on the other hand, led to the release of processed, 25- and 32-kD proteins in the conditioned media of

```
ebaf : MWPLWLCWALWVLPLAGPGAALTEEQLLASLLRQLQLSEVPVLDRADMEKLVIPAHVRAQ
lefty: MPFLWLCWALWALSLVSLREALTGEQILGSLLQQLQLDQPPVLDKADVEGMVIPSHVRTQ
       *  ******** * *      *** **.*.*** **** . ****.**.* .*** *** *

ebaf : YVVLLRR-DGDRSRGKRFSQSFREVAGRFLASEASTHLLVFGMEQRLPPNSELVQAVLRL
lefty: YVALLQHSHASRSRGKRFSQNLREVAGRFLVSETSTHLLVFGMEQRLPPNSELVQAVLRL
       ** ** .     ********* .******** ** ****************************

ebaf : FQEPVPQGALHRHGRLSPAAPKARVTVEWLV-RDDGSNRTSLIDSRLVSVHESGWKAFDV
lefty: FQEPVPRTALRRQKRLSPHSARARVTIEWLRFRDDGSNRTALIDSRLVSIHESGWKAFDV
       ******  **.*. ****   .****.***  ******** ********.**********

ebaf : TEAVNFWQQLSRPPEPLLVQVSVQREHLGPLASGAHKLVRFASQGAPA--GLGEPQLELH
lefty: TEAVNFWQQLSRPRQPLLLQVSVQREHLGPGTWSSHKLVRFAAQGTPDGKGQGEPQLELH
       ************* .***.***********     ******* ** *   * ********

ebaf : TLDLRDYGAQGDCDPEAPMTEGTRCCRQEMYIDLQGMKWAKNWVLEPPGFLAYECVGTCQ
lefty: TLDLKDYGAQGNCDPEAPVTEGTRCCRQEMYLDLQGMKWAENWILEPPGFLTYECVGSCL
       ****.******.******.************.******** **.******* ***** *

ebaf : QPPEALAFNWPFLGPRQCIASETASLPMIVSIKEGGRTRPQVVSLPNMRVQKCSCASDGA
lefty: QLPESLTSRWPFLGPRQCVASEMTSLPMIVSVKEGGRTRPQVVSLPNMRVQTCSCASDGA
       * ** *   *********.***  *******.****************** ********

ebaf : LVPRRLQHRPWCIH
lefty: LIPRRLQP------
       *.*****
```

Figure 6. Comparison of deduced amino acid sequence of ebaf with lefty. The deduced amino acid sequence of ebaf was aligned with the amino acid sequence of lefty by the CLUSTAL program (33). Gaps were introduced by the program for achieving the best alignment. Identical amino acids are shown by asterisks and conserved amino acids by dots.

the cell cultures, corresponding to cleavage at the first and second RXXR sites, respectively (24). Therefore, the processing of the protein and cleavage in the first versus the second RXXR site may be dependent on the cell type that expresses the protein.

To verify that the endometrial *ebaf* is the same as that found in the placenta, the cDNAs from several late secretory endometria were amplified with a primer set (5′ primer; TCA GCG AGG TGC CCG TAC T, 3′ primer; AGT TCT TAG AGC TGA AGC CC). The product amplified from these endometria were of the expected size (1,600 base pairs). This fragment was cloned. A stretch of 450 bases of this clone from base 189 to 639 was sequenced using the following primers: (5′ TCA GCG AGG TGC CCG TAC T 3′, 5′ TCA CGT CGA AGG CCT TC 3′ and 5′ TCA GGG TCA CAG TCG CCC 3′). The sequence of this region was identical to the sequence in the same region of the placental cDNA.

Chromosomal localization of ebaf gene. For chromosomal localization of the *ebaf* gene, 48 metaphase spreads were analyzed for the presence of FITC-labeled fluorescent signals. Specific hybridization spots on the long arm of chromosome 1s were found in 43 metaphases. The majority of these metaphases (> 75%) have revealed two signals on each chromosome (one signal on each chromatid) (Fig. 8). High-resolution

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TGFβ1   CCVRQLYIDFRKDLGW--KWIHEPKGYHANFCLGPCPYI---WSLDTQYSKVLALYNQH-----NPGASAAPCCVPQALEPLPIVYYVGRKPKV-EQL---SNMIVRSCKC--S
MIS     CALRELSVDLRAE-----RSVLIPETYQANNCQGVCGWPQSDR----NPRYGNHVVLLLKMQARGAALARPPCCVPTAYAG-KLLISLSEERISAHHV---PNMVATECGC--R
BMP-7   CKRHELYVSFR-DLGW-QDWIIAPEGYAAYYCEGECAFPLNSYMNATNHAIVQTL-----VHFINPETVPKPCCAPTQLNAISVLYFDDSSNVILKKY---RNMVVRACGC--H
INHβA   CCRRQFFVSFR-DIGW-NDWIIAPSGYHANYCEGECPSHIAG-TSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDI---QNMIVEECGC--S
GDF-3   CHPHQLFINFC-DLGW-HKWVIAPKGFMANYCHGECPFSMTIYLNSSNYAFMQAL-----MHMADPK-VPKAVCVPTKLSPISMLYQDSDKNVILKHY---EDMVVDECGC--G
GDF-9   CELHDFRLSFC-QLGW-DNWIVAPHRYNPRYCKGDCPRAVRHRYGSPVHTMVQNII--Y--EKLDP-SVPRPSCVPGKYSPLSVLTIEPDGSIAYKEY---EDMIATKCTC--R
lefty   CCRQEMYIDLQ-GMKWAENWILEPPGFLTYECVGSC----------------LQLPESLTSRWPFLGPRQ-CVASEMTSLPMIVSVKEGGRTRPQVVSLPNMRVQTCSCASD
ebaf    CCRQEMYIDLQ-GMKWAKNWVLEPPGFLAYECVGTC----------------QQPPEALAFNWPFLGPRQ-CIASETASLPMIVSIKEGGRTRPQVVSLPNMRVQKCSCASD
```

Figure 7. Comparison of the deduced amino acid sequence of ebaf and those of other members of the TGF-β superfamily. The sequences of the human transforming growth factor (TGF)-β_1 precursor, mullerian inhibiting factor precursor (MIS), bone morphogenetic protein (BMP)-7, inhibin-β A chain precursor (INH-βA) and the sequences of the mouse growth differentiation factor (GDF)-3, GDF-9, and lefty were aligned by the CLUSTAL program (33) with the sequence of the ebaf to show the conserved cysteine residues (*boxed*). Gaps were introduced by the program for achieving the best alignment.

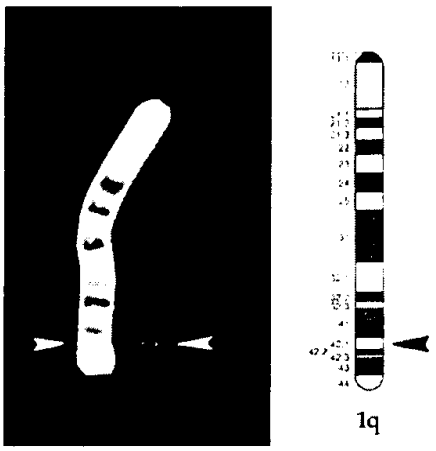

Figure 8. Chromosomal localization of *ebaf*. Partial karyotype of G-banded chromosome 1 is shown along with the same chromosome hybridized with FITC-labeled *ebaf* cDNA, indicating that this gene is mapped at 1q42.1 (*arrowhead*). Ideogram of the long arm of human chromosome 1 (G-banding at 550 bands level) shows the precise location of *ebaf* gene (*arrowhead*).

G-banding of chromosome 1s and the same chromosomes hybridized with FITC-labeled *ebaf* gene were compared to determine the location of the hybridization signals. Fig. 7 shows the physical mapping of FITC-labeled *ebaf* gene to the long arm of chromosome 1. A schematic diagram of the high-resolution G-banded chromosome 1q (550 bands) shows the precise location of the *ebaf* gene on chromosome 1, at band q42.1 (Fig. 8).

In addition to the sequence similarity, *ebaf* and *lefty* share several other features. In situ hybridization revealed that the transient expression of *ebaf* was primarily confined to the mesenchymal cells of the endometrial stroma rather than the epithelium or endothelium. The *lefty* mRNA was also transiently expressed in the mesoderm in the left half of the gastrulating mouse embryo just before the first sign of lateral symmetry appeared (24). *ebaf* gene is located on human chromosome 1. Similarly, the location of the lefty has been provisionally assigned to chromosome 1 (24). Taken together, the available data show that *ebaf* is a new member of the TGF-β superfamily. In view of chromosomal localization, great homology in the cDNA, and the predicted protein sequences and other structural features, *ebaf* may represent the human homolog of mouse *lefty*.

Steroid hormones are the systemic signals whose withdrawal leads to endometrial bleeding and tissue shedding. On the other hand, *ebaf* may be a member of the family of genes that locally participates in the expression of these characteristic attributes of human endometrium. Recent evidence suggests that expression of certain members of matrix metalloprotease family (MMP) that degrade extracellular matrix appears at defined and distinct time periods during menstrual bleeding (31, for a recent review see reference 4). These findings support the viewpoint that specific genes exist in human endometrium that locally regulate the processes leading to endometrial bleeding. *ebaf* may be a component of the molecular repertoire that locally participates in normal menstrual as well as abnormal endometrial bleeding.

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DISTINCT TUMOR SPECIFIC EXPRESSION OF TGFB4 (*ebaf*)*, A NOVEL HUMAN GENE OF THE TGF-β SUPERFAMILY

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TABLE OF CONTENTS

1. ABSTRACT

We recently identified a novel gene of the TGF-β superfamily, endometrial bleeding associated factor, TGFB4 (*ebaf*), that, throughout the menstrual cycle, exhibited a defined expression in human endometrium. Here, we report on the expression of TGFB4 (*ebaf*) in normal and neoplastic human tissues. The expression of this gene was absent in a host of normal tissues including lung, stomach, small bowel, liver, kidney, breast, lymph node, spleen, ovary and fallopian tube. However, a weak expression

of the 2.1 kb variant of the TGFB4 (*ebaf*) mRNA was observed in rectal, ovarian, and testicular tissues and the 2.1 and 2.5 kb TGFB4 (*ebaf*) mRNAs were observed in the pancreatic tissue. The expression of the mRNA of this gene was absent in sarcomas, Hodgkin's and non-Hodgkin's lymphomas, melanomas, squamous cell carcinomas, hepatocellular carcinomas, renal cell carcinomas, and adenocarcinomas of the breast, endometrium and lung. The expression of the TGFB4 (*ebaf*) mRNA was observed primarily in adenocarcinomas that exhibited a mucinous differentiation. This included colonic, duodenal, and ovarian adenocarcinomas. The expression of TGFB4 (*ebaf*) mRNA was absent in non-mucinous colonic, gastric and ovarian adenocarcinomas and adenocarcinomas of colon metastatic to the liver. However, some serous adenocarcinomas of the ovary also exhibited TGFB4 (*ebaf*) mRNA. The testicular tumors, seminomas and embryonal carcinomas, also expressed TGFB4 (*ebaf*) mRNA. These findings show that the TGFB4 (*ebaf*) mRNA has distinct tumor specific expression.

2. INTRODUCTION

Some human genes such as β-actin and GAPDH mRNAs are present in virtually every cell (1-2). These genes, in view of their abundance, are considered part of the housekeeping gene repertoire. Some other genes are expressed in distinct tissues. For example, thyroglobulin or prostate specific antigen are, respectively, expressed in the thyroid and prostate (3-4). A different set of genes are expressed in cells of distinct lineages. For example, various isoforms of cytokeratin are expressed in the epithelial cells (5-7), vimentin is expressed in the mesenchymal and lymphoid cells (7-11), LCA in the lymphoid cells (12), desmin in the muscle cells (9-11), and neurofilament in the glial cells (9-11). Such a confined gene expression is shared by tumors derived from these tissues allowing the gene product to be used as a tumor marker. Among the most commonly used tumor markers is carcinoembryonic antigen (13). Its serum level is primarily used for the detection of gastrointestinal tumors. The serum level of the CA125 is used in the diagnosis of ovarian tumors and that of prostate specific antigen in the detection of the prostate cancers (4,13). We recently identified a novel member of the TGF-β superfamily, endometrial bleeding associated factor (*ebaf*), which was expressed in the endometrium (15). According to the guidelines of the Human Gene Nomenclature (http://www.gene.ucl.ac.uk/nomenclature/guidelines.html#Heading6), *ebaf* has been designated as TGFB4. The expression of the TGFB4 (*ebaf*) mRNA in endometrium was confined to the late secretory and menstrual phases and was seen in endometria with active bleeding (15). This gene was not expressed in endometrium during proliferative or early or mid-secretory phases of the menstrual cycle (15). *In situ* hybridization revealed that the gene was expressed primarily in the stroma and rarely in the endometrial glands. Endothelial cells failed to express the gene (15). The expression of the *lefty*, the mouse homolog of the TGFB4 (*ebaf*), also showed a narrow tissue-specific distribution. Meng *et al*, by *in situ* hybridization showed that the expression of *lefty* was found only in the mesenchymal cells of the mouse embryo on the left side of the body (16). The expression of *lefty* disappeared shortly after birth (16).

These findings show that the expression of this gene is exquisitely controlled in the body, both in the mouse and in the human. Therefore, to gain an insight on the tissue distribution of the TGFB4 (*ebaf*) mRNA, in this study, we carried out Northern blot analysis on a panel of normal human tissues. In addition, we also examined the TGFB4 (*ebaf*) mRNA expression in tumors derived from epithelia, melanocytes and mesenchyme including adenocarcinomas, squamous cell carcinomas, melanomas, lymphomas and various types of sarcomas.

3. MATERIALS AND METHODS

3.1 Materials

A 1.1 kb cDNA fragment of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was obtained from Clontech (Palo Alto, CA). Deoxycytidine 5' tri-phosphate dCTP α-^{32}P (3000 Ci/mmol) was from Dupont NEN Research Products (Boston, MA). Prime-a-Gene labeling kit was from Promega (Madison, WI). RNA STAT-60TM was from Tell-Test, Inc (Friendwood, TX). Silane-coated, RNAse free, slides coated (Silane-PrepTM) for *in situ* hybridization and the Kodak-OMAT films were obtained from Sigma Chemical Company (St Louis, MO). Nick columns were obtained from Pharmacia Biotech (Piscataway, NJ). Digoxygenin labeling kit (SP6/T7) and DIG nucleic acid detection kit were from Boehringer Mannheim Corporation (Indianapolis, IN). All other chemicals were from either Sigma Chemical Company or Fisher Scientific (Pittsburgh, PA).

3.2 Processing of tissues

Tissues were obtained from the tumor bank at the H. Lee Moffitt Cancer Center according to the rules and regulations of the institution. Tumor tissues and, when available, normal tissues surrounding the tumor were frozen in liquid nitrogen and maintained at -70^{O}C until used. Samples of the normal tissues and tumors were embedded in paraffin, sectioned and stained by hematoxylin and eosin for light microscopic examination and for establishing the diagnosis and determination of the tumor type.

3.3 Isolation of RNA and Northern blotting

RNA was isolated by acid guanidinium thiocyanate-phenol-choloroform extraction method as described (17). Briefly, the tissues were homogenized in RNA STAT-60^{TM}. Each, 50-100 mg of tissue was homogenized in 1 ml of RNA STAT-60^{TM} in a glass or Teflon Dounce homogenizer. Each homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added for each ml of RNA STAT-60^{TM}used. Each sample was covered and shaken vigorously for 15 seconds and allowed to stand at room temperature for 2-3 min. Following centrifugation at 12,000xg for 15 min at 4^{o}C, each homogenate was separated into a lower phenol/chloroform phase and an upper aqueous phase. RNA in the upper aqueous phase was transferred

to fresh tubes and mixed with isopropoanol to precipitate the total RNA. After centrifugation and drying, the precipitated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by vigorous pipetting and by a gentle heating at 55°-60°C. The amount of RNA in each sample was determined spectrophotometrically. The quality of RNA was judged by the integrity of ribosomal. Northern blotting was done as described (18). Briefly, 20 μg of total RNA of each sample was denatured at 65°C in a RNA loading buffer, electrophoresed in 1% agarose containing 2.2 M formaldehyde gel, and blotted onto a Hybond nylon membrane using a positive pressure transfer apparatus (Posiblot, Stratagene, La Jolla, CA). The RNA was fixed to the membrane by UV crosslinking. Using the Prime-a-Gene kit, cDNA was labeled with [^{32}P] to a high specific activity, and purified by Nick columns. Membranes were prehybridized in 50% formamide, 10x Denhardt's solution, 4% saline sodium citrate (SSC), 0.05 M sodium pyrophosphate and 0.1 mg/ml of denatured Hering sperm DNA at 42°C for 2-4 hr and hybridized for 16 hr at 42°C with 106 cpm/ml of heat-denatured probe in the same buffer containing 10% dextran sulphate. Then, membranes were sequentially washed three times in 4x SSC, one time in 0.5x SSC and then one time in 0.1x SSC. All washes contained 0.1% sodium dodecyl sulphate (SDS), and were done at 65°C for 20 min each. The membranes were subjected to autoradiography at -70°C with intensifying screens. The same blot was stripped and reprobed for GAPDH. To reprobe a blot, the probe was stripped from the membrane in 75% formamide, 0.1x saline sodium phosphate ETDA (SSPE), and 0.2% SDS at 50°C for one hour.

3.4 In situ hybridization

Digoxigenin-labeled sense and anti-sense RNAs of TGFB4 (*ebaf*) were synthesized by *in vitro* transcription of the, full length cDNA, cloned into pBluescript^{R} SK^{-} using digoxygenin dUTP. After alkaline hydrolysis, the probes were subjected to agarose gel electrophoresis to determine the size of the digested RNA fragments. Dot blotting was performed on the RNA fragments to insure that they were labeled. *In situ* hybridization was performed as previously described (19-20) Briefly, frozen sections of endometria were mounted on silane-coated, RNAse-free, slides and fixed in 4% formalin in PBS for 15 min at 4°C. The tissue sections were rinsed in 2xSSC and then treated with proteinase K (1 μg/ml in 0.1 M Tris, 50 mM EDTA, 20 min, 37°C) and acetylated for 10 min in 0.1 triethanolamine (pH 8.0), 0.9% sodium chloride and 0.25% acetic anhydride. The slides were dipped once in 2x SSC and then were dehydrated in ascending series of ethyl alcohol and air dried. The slides were prehybridized for 1 hr at 37°C in 50% formamide, 1x Denhardt's solution and 500 μg/ml tRNA, 0.3 M sodium chloride, 10 mM Tris, 1 mM EDTA (pH 8), and 10% dextran sulfate. Then, sections were incubated at 55°C overnight in the same solution containing the appropriate concentration of the probe. The amounts of labeled probes needed were empirically determined first by a series of *in situ* hybridization experiments using various dilutions of the probes. Sense probe was used as the control. After hybridization, slides were washed

three times for 10 min each at room temperature in 2x SSC, and the excess SSC was removed. The sections were then incubated with RNAse A (20 μg/ml) in 500 mM NaCl, 1 mM EDTA and 10 mM Tris HCl pH 8 at 37°C for 30 min to remove the non-hybridized RNA. The sections were washed three times at room temperature, for 15 min each, in 2x SSC, 1xSSC, and 0.5x SSC and a final wash in 0.1x SSC at 55°C for 45 min. Slides were washed in 100 mM Tris (pH 8), 150 mM sodium chloride for 10 min. Then, sections were blocked in 5% normal horse serum in the same buffer for 20 min at 37°C. Slides were incubated with alkaline phosphatase labeled, anti-digoxygenin antibody for 1 hr at 37°C, washed and developed in a mixture of Nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

4. RESULTS

We first examined the expression of the TGFB4 (*ebaf*) mRNA in normal tissues (Fig 1, Table 1). RNAs from several normal late secretory and menstrual endometrial tissues were used as control in these experiments (Fig 1-5). As reported previously (15), the prominent TGFB4 (*ebaf*) mRNA exclusively expressed in endometrium during the late secretory and menstrual phases was 2.5 kb (Fig 1). However, additional, smaller in size mRNAs were also noted in the menstrual endometria (Fig 3, Fig 5). These mRNAs were 2.1 and 1.5 kb in size (Fig 3, Fig 5). From a large number of tissues tested, the TGFB4 (*ebaf*) mRNA was expressed only in the ovary, rectum, testis and pancreas (Fig 1, Table 1). Both 2.5 and 2.1 kb TGFB4 (*ebaf*) mRNAs were expressed in the pancreas, whereas the TGFB4 (*ebaf*) mRNA, weakly expressed, in the rectum, in an ovary and testis, was 2.1 kb (Fig 1, Table 1). TGFB4 (*ebaf*) mRNA was not expressed in the breast, stomach, small bowel, colon, kidney, lung, fallopian tube, spleen and lymph node (Fig 1, Table 1).

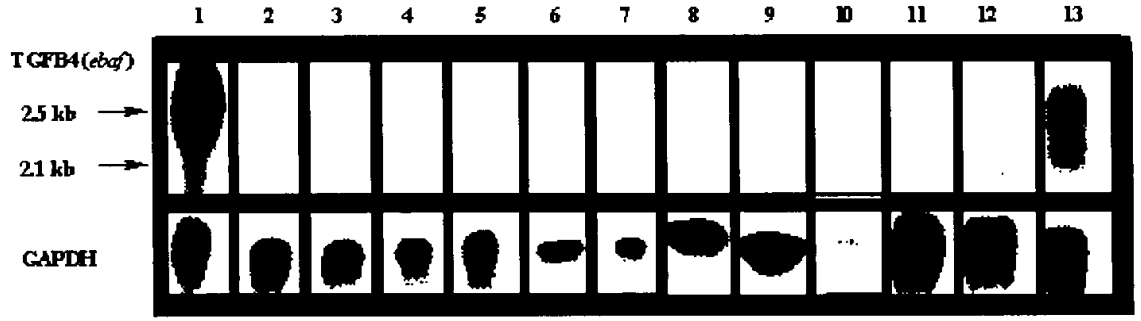

Fig 1. Northern blot analysis of TGFB4 (*ebaf*) mRNA in normal tissues. 20 μg total RNA from each tissue (lane 1: normal menstrual endometrium serving as the positive control) and other normal tissues

(lane 2: spleen, lane 3: lymph node, lanes 4 and 5: stomach, lane 6: lung, lane 7: breast, lane 8: liver, lane 9 and 10: ovary, lane 11: rectum, lane 12: testis, lane 13: pancreas) was subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel). The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blots with a cDNA probe to GAPDH (lower panel). As shown, bands of TGFB4 (*ebaf*) mRNA in the size of 2.1 and 2.5 kilobase (kb) are detected in the endometrium. A weak 2.1 kb TGFB4 (*ebaf*) mRNA is detected in the ovary, rectum, and testis. In the pancreas, both the 2.1 and 2.5 kb TGFB4 (*ebaf*) mRNA are detected.

Table 1. Expression of TGFB4 (*ebaf*) mRNA in normal human tissues.

TISSUE	NUMBER OF TISSUES	NORTHERN BLOT FINDING
Breast	5	-
Stomach	3	-
Small Bowel	1	-
Colon	11	-
Rectum	2	2.1 kb
Liver	6	-
Pancreas	2	2.1 and 2.5 kb
Kidney	1	-
Lung	1	-
Fallopian Tube	1	-
Ovary	7	-

Ovary	1	2.1 kb
Testis	2	2.1 kb
Spleen	1	-
Lymph node	1	-

We then examined the TGFB4 (*ebaf*) mRNA expression in the cancers derived from cells of different lineages. In eleven adenocarcinomas of colon, adjacent normal colonic tissues, non-involved by the tumor were available for the study. The RNAs from the neoplastic and surrounding normal tissues were both subjected to the Northern blot analysis for the detection of the TGFB4 (*ebaf*) mRNA (Fig 2, Table 2).

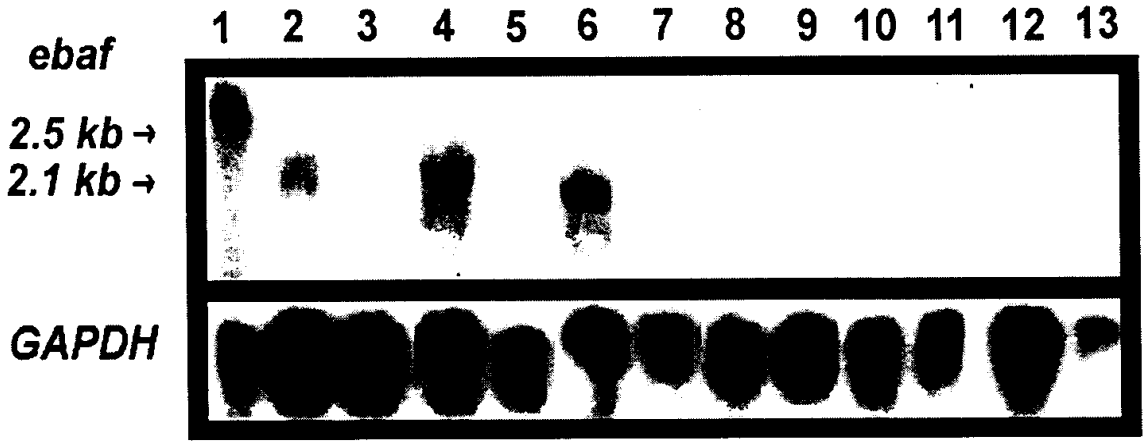

Figure 2. Northern blot analysis of TGFB4 (*ebaf*) mRNA in colonic adenocarcinomas. 20 μg total RNAs from a normal late secretory endometrium which served as the positive control (lane 1) as well as mucinous adenocarcinomas of colon (lanes 2, 4, 6), non-mucinous adenocarcinomas of colon (lanes 8, 10, and 12) and adjacent normal colon (lanes 3, 5, 7, 9, 11 and 13) were subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel). A 2.1 kb TGFB4 (*ebaf*) mRNA is detected in the colonic adenocarcinomas with mucinous differentiation and not those which did not exhibit a mucinous differentiation or the adjacent normal colonic tissues. The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not

shown) and hybridization of the blots with a cDNA probe to GAPDH (lower panel).

Table 2. Expression of TGFB4 (*ebaf*) mRNA in human tumors

TUMOR TYPE	NUMBER OF TUMORS	NORTHERN BLOT FINDING
Mucinous adenocarcinoma of colon*	7	2.1 kb
Mucinous adenocarcinoma of colon*	4	-
Mucinous adenocarcinoma of the duodenum	1	2.1 kb
Mucinous adenocarcinoma of the ovary	3	2.1 kb
Serous cystadenocarcinoma of ovary	2	2.1 kb
Serous cystadenocarcinoma of ovary	2	2.5 kb
Serous cystadenocarcinoma of ovary	5	-
Non-mucinous colonic adenocarcinoma metastatic to ovary	1	-
Non-mucinous adenocarcinoma of colon	7	-
Non-mucinous adenocarcinoma of	1	-

uterine cervix		
Non-mucinous adeoncarcinoma of the stomach	3	-
Endometrioid adenocarcinoma of ovary	1	-
Hepatocelluar carcinoma	3	-
Renal Cell Carcinoma	3	-
Liver metastasis; consistent with colonic primary*	6	-
Adenocarcinoma of lung	7	-
Adenocarcinoma of breast*	5	-
Adenocarcinoma of endometrium	3	-
SCC of the Larynx	1	-
SCC of the Lung	4	-
SCC of the Uterine cervix	1	-
Teratoma-embryonal cell carcinoma	1	2.5 kb
Germ cell tumor-embryonal cell carcinoma	1	2.5 kb
Seminoma	2	2.1 kb
Seminoma	1	-

Leiomyosarcoma, gastric	1	-
Leiomyosarcoma, colon	1	-
Leiomyosarcoma, pelvic	1	-
Chondrosarcoma, thoracic wall	1	-
Osteosarcoma, metastatic to the lung	3	-
Liposarcoma, retroperitoneum	1	-
Synovial sarcoma, metastatic to the chest wall	1	-
Synovial sarcoma, parotid	1	-
Synovial sarcoma, leg	1	-
Angiosarcoma, mediastinal	1	-
Lymphoma	1	-
Lymphoma, B cell type	1	-
Lymphoma, B cell, spleen	1	-
Lymphoma, T cell, groin	1	-
Lymphoma, T cell, angiocentric, hip	1	-
Hodgkin's disease, mixed cell type, lymph node	1	-
Melanoma	5	-

*: Normal tissues around the tumors were available for the Northern blot analysis and did not exhibit TGFB4 (*ebaf*) mRNA

Whereas the TGFB4 (*ebaf*) mRNA was not detected in the normal colon, the expression of the 2.1 kb TGFB4 (*ebaf*) mRNA was detected in seven of the eleven cases of adenocarcinomas of colon (Fig 2, Table 2). The histologic evaluation of the positive cases revealed these cases to have a mucinous differentiation. Similarly, an adenocarcinoma of the duodenum and three cases of mucinous adenocarcinomas of the ovary that also exhibited mucinous differentiation expressed the 2.1 kb TGFB4 (*ebaf*) mRNA (Fig 3, Table 2).

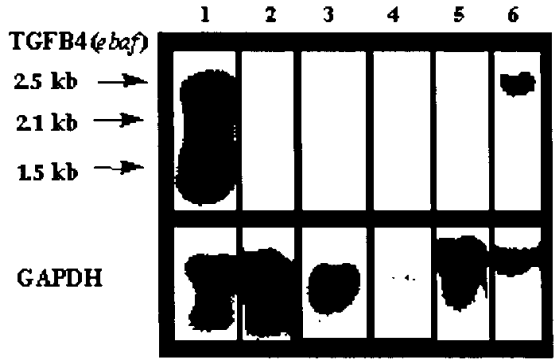

Figure 3. Northern blot analysis of TGFB4 (*ebaf*) mRNA in ovarian adenocarcinomas. 20 µg total RNA from a normal menstrual endometrium which served as the positive control (lane 1) as well as other tumors (lanes 2-4, serous cystadenocarcinomas; lane 5: endometrioid adenocarcinoma; lane 6, mucinous cystadenocarcinoma) was subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel). There is relatively more TGFB4 (*ebaf*) mRNAs in the menstrual endometrium than the tumor tissues leading to overexposure of the band of TGFB4 (*ebaf*) mRNAs in endometrium. The 2.5 kb TGFB4 (*ebaf*) mRNA is detected in serous (lanes 3 and 4) and mucinous cystadenocarcinomas (lane 6) of the ovary. The 2.1 kb mRNA is detected in serous cystadenocarcinoma (lane 2). The endometrioid adenocarcinoma of the ovary does not exhibit TGFB4 (*ebaf*) mRNA. The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blot with a cDNA probe to GAPDH (lower panel).

The non-mucinous adenocarcinomas, whether primary in the colon, metastatic to the liver, or the ovary, did not express TGFB4 (*ebaf*) mRNA (Table 2). In addition, in colonic adenocarcinomas metastatic to the liver, the adjacent liver, non-involved by the tumor, also did not show any evidence of TGFB4 (*ebaf*)

mRNA expression (Table 1). In the ovary, besides the mucinous adenocarcinomas some serous adenocarcinomas (n=4/9) also expressed the TGFB4 (*ebaf*) mRNA (Table 2). In two cases, the TGFB4 (*ebaf*) mRNA was 2.1 and in two cases 2.5 kb (Fig 3, Table 2). On the other hand, other adenocarcinomas of ovary including those exhibiting endometrioid differentiation failed to express TGFB4 (*ebaf*) mRNA (Fig 3). To localize the cells that express the TGFB4 (*ebaf*) mRNA in the adenocarcinomas of colon and ovary, *in situ* hybridization was carried out on the positive cases. As a positive control, tissue sections from a late secretory endometrium, was used. As reported previously (15), the hybridization signal was noted primarily in the endometrial stroma and endometrial glands or the endothelium did not show presence of signal (Fig 4A). Only rarely, few glands close to the surface epithelium exhibited a positive hybridization signal (data not shown). Hybridization with the sense RNA did not produce any signal in the normal endometrium (Fig 4B). In tumor tissue sections hybridized with the anti-sense TGFB4 (*ebaf*) RNA, there was some minimal expression of TGFB4 (*ebaf*) mRNA in the tumor stroma. However, by far the most prominent expression of TGFB4 (*ebaf*) mRNA was noted in the neoplastic epithelial cells both in the colon (Fig 4C) and in the ovary (Fig 4E). The sense probe of the TGFB4 (*ebaf*) failed to show a hybridization signal in the same tissues (Fig 4D, 5F). In the adenocarinomas of other organs such as breast, lung, pancreas, cervix, stomach, liver, kidney and endometrium, TGFB4 (*ebaf*) mRNA was not detectable by the Northern blot analysis (Table 2).

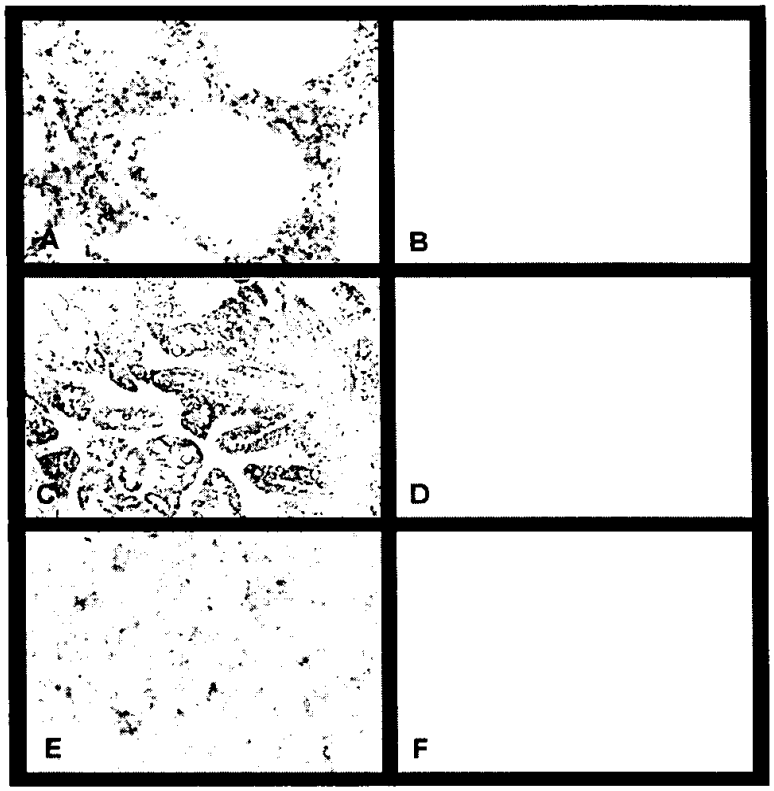

Fig 4. *In situ* hybridization of TGFB4 (*ebaf*) mRNA in adenocarcinomas of colon and ovary. Digoxigenin-labeled anti-sense (A, C, E) and sense RNAs (B, D, F) of TGFB4 (*ebaf*) were synthesized by *in vitro* transcription of the full length TGFB4 (*ebaf*) cDNA cloned into pBluescript^{R} SK(+/-). Sections of a late secretory endometrium were used as the positive control (A). Hybridization signal is seen in the endometrial stroma (A) and in the epithelial cells of a colonic adenocarcinoma with mucinous differentiation (C) and ovarian adenocarcinoma (E). The sense probe did not reveal any hybridization signal in the sections of the endometrium (B) or the same tumors (D, F).

Five cases of testicular cancer were examined for TGFB4 (*ebaf*) mRNA expression by the Northern blot analysis. Two cases that showed an embryonal carcinoma component exhibited the 2.5 kb TGFB4 (*ebaf*) mRNA. On the other hand, two out of three cases of seminomas expressed the 2.1 kb TGFB4 (*ebaf*) mRNA (Fig 5).

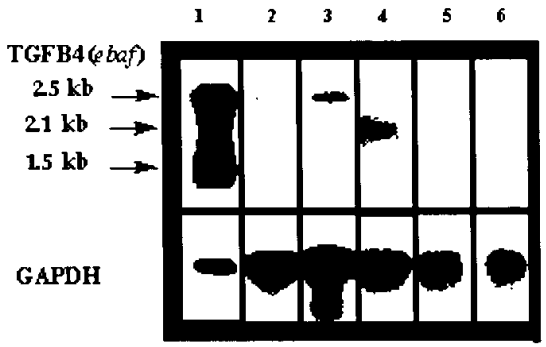

Fig 5. Northern blot analysis of TGFB4 (*ebaf*) mRNA in testicular cancers. 20 μg total RNA from a normal menstrual endometrium which served as the positive control (lane 1) and each tumor tissue (lane 2: teratoma-embryonal cell carcinoma, lane 3: mixed germ cell tumor containing embryonal carcinoma, lanes 4-6; seminoma) was subjected to the Northern blot analysis using the entire placental-derived TGFB4 (*ebaf*) cDNA as the probe (upper panel).). The blot was exposed for long duration to detect TGFB4 (*ebaf*) mRNA in the neoplastic tissues. This resulted in the overexposure of the TGFB4 (*ebaf*) mRNAs detected in the endometrium. The 2.5 kb TGFB4 (*ebaf*) mRNA is detected in the tumors containing embryonal carcinoma. The 2.1 kb mRNA is detected in two of three cases of seminoma. The integrity of RNA and equal loading was verified by staining the 18S and 28S ribosomal RNAs (not shown) and hybridization of the blots with a cDNA probe to GAPDH (lower panel).

The Northern blot analysis of squamous cell carcinomas derived from larynx, lung and uterine cervix did not show any evidence of the TGFB4 (*ebaf*) mRNA expression (Table 2). Furthermore, the TGFB4 (*ebaf*) mRNA was not detectable in the non-epithelial tumors including sarcomas such as leiomyosarcoma, chondrosarcoma, osteosarcoma, liposarcoma, synovial sarcoma, as well as Hodgkin's and non-Hodgkin's lymphomas, or melanomas (Table 2).

5. DISCUSSION

In the present report, we defined the pattern of expression of TGFB4 (*ebaf*) mRNA in normal tissues. In addition to the endometrium, the 2.1 and 2.5 kb TGFB4 (*ebaf*) mRNAs were expressed in the pancreas and the expression of the 2.1 kb TGFB4 (*ebaf*) mRNA in the rectum and testis was weak and was rarely noted in the ovary. The weak to lack of expression of TGFB4 (*ebaf*) mRNA may be due to the proportionally low number of cells that express TGFB4 (*ebaf*) in these tissues. However, in contrast to the endometrium that primarily exhibits the 1.5 kb, 2.1 kb and 2.5 kb TGFB4 (*cbaf*) mRNAs, the major TGFB4 (*ebaf*) mRNA species expressed in these tissues was the 2.1 kb in size. Sequencing of the TGFB4

(*ebaf*) cDNAs obtained from normal endometria, revealed that the underlying basis for the difference in the sizes of TGFB4 (*ebaf*) mRNAs were attributable to the deletion of parts of the coding sequence of TGFB4 (*ebaf*) (unpublished data). Therefore, TGFB4 (*ebaf*) mRNA seems to exist as spliced variants. In addition, TGFB4 (*ebaf*) mRNA seems to have a distinct tissue distribution pattern. *In situ* hybridization revealed that in endometrium, TGFB4 (*ebaf*) mRNA expression was primarily confined to the endometrial stroma (15). Few, if any, endometrial glands expressed TGFB4 (*ebaf*) mRNA and the endometrial endothelial cells did not express TGFB4 (*ebaf*) mRNA (15). The expression of *lefty*, the mouse homolog of the TGFB4 (*ebaf*), was also limited to the mesenchymal cells in the mouse embryos (16). This expression was polarized and during embryogenesis was seen only on the left side of the body (16). Furthermore, the expression of *lefty* mRNA quickly disappeared postnatally. These findings show that TGFB4 (*ebaf*) mRNA has distinct tissue and cell specific expression in the embryo and in the adult tissues. It is conceivable that different splice variants of TGFB4 (*ebaf*) mRNA may exist in different cell types and lineages.

Northern blot analysis of tumors derived from different cell lineages confirmed the specific tissue distribution of the TGFB4 (*ebaf*) mRNA. TGFB4 (*ebaf*) mRNA was not expressed in tumors derived from lymphoid cells such as T and B cell lymphomas, and Hodgkin's disease. Tumors derived from endothelial cells, smooth muscle, bone, cartilage, synovium and melanocytes also did not express TGFB4 (*ebaf*) mRNA. The TGFB4 (*ebaf*) mRNA was expressed in adenocarcinomas of colon, ovary and testis. The type of the tumor in the colon and ovary that expressed the gene was derived from glandular structures of these tissues, namely adenocarcinomas. *In situ* hybridization showed that the TGFB4 (*ebaf*) mRNA was expressed primarily in the neoplastic glands rather than the tumor stroma. Histologic evaulation of adenocarcinomas expressing the TGFB4 (ebaf) mRNA showed that these tumors exhibit mucinous differentiation. In the testis, both the seminomas and tumors that contained an embryonal carcinoma component exhibited TGFB4 (*ebaf*) mRNA expression. However, the TGFB4 (*ebaf*) mRNA that was expressed in the seminomas was different in size from that expressed in the embryonal carcinomas. This difference may be attributable to the cell lineage of these tumors. Taken together, these findings show that TGFB4 (*ebaf*) mRNA is expressed in tumors of specific types and cell lineages. The difference in the expression of the 2.5 versus the 2.1 kb of TGFB4 (*ebaf*) mRNA may be dependent on the cell type that expresses the gene.

The predicted protein sequence of TGFB4 (ebaf) showed homology with and structural features of the members of TGF-β superfamily (15). The members of the TGF-β superfamily are synthesized as prepro-proteins which are cleaved at RXXR site to release the mature form of the protein. The predicted protein of TGFB4 (ebaf) exhibits two such RXXR sites which are located respectively at amino acid residues of 73-76 and 131-134 (15). The deduced amino acid sequence of lefty also contained two potential cleavage sites at amino acid residues of 74-77 and 132-135 (15). Therefore, TGFB4 (ebaf) gene products may be

secreted by the tumor cells and may be released into the peripheral circulation. The confined expression of TGFB4 (ebaf) to tumors of distinct phenotype, therefore, will make detection of TGFB4 (*ebaf*) mRNA or its protein a useful tumor marker. Tumor markers in the blood include glycoproteins secreted by the solid tumors as well as those expressed on the cell surface. The most successful markers for the diagnosis of solid tissue cancers have been α-fetoprotein and prostate specific antigen (14). The serum level of markers such as CEA and a number of carbohydrate epitopes, e.g., CA 15.3, CA 19.9, CA 50, CA 242, and mucin epitopes, such as MCA, CA 125, and DU-PAN-2 are now being used to determine the prognosis of a variety of cancers and to monitor their response to therapy (13-14). If secreted, presence of TGFB4 (ebaf) in the peripheral circulation may be potentially used for screening, diagnosis, prognosis, and monitoring of the treatment, or the detection of relapse in tumors expressing the gene.

In summary, in the present report, we defined the expression of TGFB4 (*ebaf*) mRNA in normal and neoplastic tissues. In normal tissues, the expression of TGFB4 (*ebaf*) was limited to the endometrium, and only a weak expression of TGFB4 (*ebaf*) mRNA was detectable in the rectum, ovary and testis. In the pancreas, the TGFB4 (*ebaf*) mRNA was detectable. In the neoplastic tissues, the expression of the TGFB4 (*ebaf*) mRNA was restricted to tumors of colon, ovary and testis and tumors of lymphoid, melanocytic, and mesenchymal origins did not express the TGFB4 (*ebaf*) mRNA. These findings show that TGFB4 (*ebaf*) mRNA has a narrow and distinct tissue distribution in both normal and neoplastic tissues.

6. ACKNOWLEDGMENT

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